JIPB Journal of Integrative Plant Biology

The *Arabidopsis CROWDED NUCLEI* genes regulate seed germination by modulating degradation of ABI5 protein

Wenming Zhao[1,2†], Chunmei Guan[1], Jian Feng[1,2††], Yan Liang[1†††], Ni Zhan[1,2], Jianru Zuo[1] and Bo Ren[1*]

[1]State Key Laboratory of Plant Genomics and National Plant Gene Research Center, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China, [2]University of Chinese Academy of Sciences, Beijing 100049, China. [†]Current address: Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China, [††]Current address: John Innes Centre, Norwich NR4 7UH, United Kingdom, [†††]Current address: Institute of Biotechnology, Zhejiang University, Hangzhou 310058, China. *Correspondence: bren@genetics.ac.cn

Research Article

Abstract In *Arabidopsis*, the phytohormone abscisic acid (ABA) plays a vital role in inhibiting seed germination and in post-germination seedling establishment. In the ABA signaling pathway, ABI5, a basic Leu zipper transcription factor, has important functions in the regulation of seed germination. ABI5 protein localizes in nuclear bodies, along with AFP, COP1, and SIZ1, and was degraded through the 26S proteasome pathway. However, the mechanisms of ABI5 nuclear body formation and ABI5 protein degradation remain obscure. In this study, we found that the *Arabidopsis* CROWDED NUCLEI (CRWN) proteins, predicted nuclear matrix proteins essential for maintenance of nuclear morphology, also participate in ABA-controlled seed germination by regulating the degradation of ABI5 protein. During seed germination, the *crwn* mutants are hypersensitive to ABA and have higher levels of ABI5 protein compared to wild type. Genetic analysis suggested that *CRWN*s act upstream of *ABI5*. The observation that CRWN3 colocalizes with ABI5 in nuclear bodies indicates that CRWNs might participate in ABI5 protein degradation in nuclear bodies. Moreover, we revealed that the extreme C-terminal of CRWN3 protein is necessary for its function in the response to ABA in germination. Our results suggested important roles of CRWNs in ABI5 nuclear body organization and ABI5 protein degradation during seed germination.

Keywords: ABI5; *Arabidopsis*; CROWDED NUCLEI; germination; nuclear body
Citation: Zhao W, Guan C, Feng J, Liang Y, Zhan N, Zuo J, Ren B (2015) The *Arabidopsis CROWDED NUCLEI* genes regulate seed germination by modulating degradation of ABI5 protein. **J Integr Plant Biol** XX:XX–XX doi: 10.1111/jipb.12448
Edited by: Wei-Cai Yang, Institute of Genetics and Developmental Biology, CAS, China
Received Sept. 8, 2015; **Accepted** Nov. 10, 2015
Available online on Nov. 13, 2015 at www.wileyonlinelibrary.com/journal/jipb

INTRODUCTION

The ability to generate seeds improves plant dispersal and survival under unfavorable conditions. Under permissive environmental conditions, seed dormancy breaks and germination occurs. Seed germination is strictly controlled by various external and internal cues, including light, temperature, osmotic stress, glucose and phytohormones (Bewley 1997; Lopez-Molina et al. 2001, 2002; Penfield et al. 2005; Dekkers et al. 2008). Among these endogenous signals, the phytohormones abscisic acid (ABA) and gibberellin (GA) play central and antagonistic roles in the regulation of seed germination. GA promotes seed germination, whereas ABA induces seed maturation and dormancy and inhibits seed germination (Karssen et al. 1983; Karssen et al. 1989; Kucera et al. 2005; Nambara and Marion-Poll 2005).

Genetic screens using germination assays in *Arabidopsis* have identified several factors involved in ABA signal transduction, including ABA INSENSITIVE 1 (ABI1) to ABI5 (Koornneef et al. 1984; Finkelstein 1994, 1998). ABI1 and ABI2 are Ser/Thr protein phosphatase 2C (PP2C) proteins that negatively regulate SNF1-related protein kinase 2 (SnRK2) proteins and thus regulate the downstream ABA-responsive *cis*-acting elements (AREB/ABFs) (Kobayashi et al. 2005; Umezawa et al. 2009). ABI3, ABI4 and ABI5 belong to the B3, AP2 and bZIP transcription factor families, respectively, and play important roles in seed germination and early seedling establishment (Parcy et al. 1994; Finkelstein 1998, 2000). ABI5 functions as a positive regulator in the ABA signaling pathway and can bind to AREBs in the promoters of ABA-responsive genes and activate gene expression (Finkelstein and Lynch 2000; Lopez-Molina and Chua 2000). Genetic evidence showed that *ABI5* acts downstream of *ABI3* (Lopez-Molina et al. 2002). ABI5 protein levels directly affect ABA-dependent seed germination and post-germination growth (Lopez-Molina et al. 2001; Brocard et al. 2002; Guan et al. 2014). This ABA-dependent regulation of ABI5 occurs within a narrow developmental window following germination. *ABI5* transcripts and ABI5 protein decrease very rapidly to undetectable levels after stratification (Lopez-Molina et al. 2001, 2002). ABI5 protein is degraded through the 26S proteasome pathway, associated with the E3 ubiquitin ligase KEEP ON GOING (KEG) and with two components of the CUL-DDB1-DWD (Cullin4-Damaged DNA Binding1-DDB1 binding WD40) E3 ligase complex, DWA1 and DWA2 (DWD hypersensitive to ABA1 and 2) (Stone et al. 2006; Lee et al. 2010; Liu and Stone 2010). ABI FIVE BINDING PROTEIN (AFP) also regulates ABI5 protein degradation. The colocalization of ABI5, AFP and

COP1, an important component of the E3 ligase complex, in nuclear bodies (NBs) indicates that AFP may mediate ABI5 degradation through the ubiquitin pathway in NBs (Lopez-Molina et al. 2003).

NBs, discrete functional subcompartments in the nuclei of animal and plant cells, are believed to be where protein turnover, gene expression, DNA repair and other biological processes occur in cells (Shaw and Brown 2004; Spector 2006). Plants have NBs containing some phytochrome receptors, including PHYA to PHYE, CRY1, CRY2, and UV8. These phytochrome receptors can localize to the NBs shortly after activation by the appropriate wavelength of light (Kleiner et al. 1999; Yamaguchi et al. 1999; Kircher et al. 2002; Favory et al. 2009; Yu et al. 2009). Some components of the E3 ubiquitin ligase complex, including COP1 and SPA1, colocalize with these phytochrome receptors, indicating that the degradation of these phytochrome receptors occurs in the NBs (Wang et al. 2001; Lian et al. 2011; Liu et al. 2011; Zuo et al. 2011; Gu et al. 2012).

Plants also have phytohormone-related NBs. For example, auxin causes IAA17, a negative regulator of the auxin signaling pathway, to be recruited into proteolytic NBs, which also contain some components of the SCF, COP9 signalosome, and 26S proteasome (Tao et al. 2005). ABI5, the key positive regulator in the ABA pathway, and its SUMO E3 ligase SIZ1 colocalize in the NBs, which also contain AFP and COP1 (Lopez-Molina et al. 2003; Miura et al. 2005; Catala et al. 2007; Miura et al. 2009). However, how these NBs form and how the target proteins get degraded in NBs remain poorly understood in plants.

In mammals, much work has examined NBs involved in diseases. For example, the morphology of promyelocytic leukemia (PML) nuclear bodies dramatically changes in acute promyelocytic leukemia patients (Daniel et al. 1993; Dyck et al. 1994; Koken et al. 1994; Weis et al. 1994). PML NBs may recruit related proteins to enter the NBs where they get modified, activated, compartmentalized or degraded. To date, more than 100 proteins have been identified in PML NBs, including proteasome protein, ubiquitin, PML and lamin (Lallemand-Breitenbach and de The 2010).

PML protein, which has a coiled-coil domain, functions as the "organizer" of the PML NBs. The first step of the organization of NBs is the conjugation of PML and the nuclear matrix (Müller et al. 1998; Lallemand-Breitenbach et al. 2001). The formation of NBs requires nuclear matrix proteins, such as lamin, which also contain coiled-coil domains. Although plants have no lamin, the coiled-coil domain proteins, Nuclear Matrix Constituent Protein 1 (NMCP1) in carrot and the related CROWDED NUCLEI (CRWN) proteins, which were originally designated LITTLE NUCLEI (LINCs), in *Arabidopsis* were predicted to have similar functions to lamin A (Masuda et al. 1997; Dittmer et al. 2007).

Arabidopsis has four CRWN proteins, CRWN1 to CRWN4, which all localize in the nucleus. CRWN proteins play redundant, essential roles in the regulation of nuclear structure, as most of the *crwn* single mutants have no obvious phenotype but the double mutants display reduced nuclear size, abnormal nuclear shape, and aberrant heterochromatin organization. The *crwn* triple mutants have severe defects in growth and development, and some have a lethal phenotype (Dittmer et al. 2007; Sakamoto and Takagi 2013; Wang et al. 2013). CRWN1 and CRWN2, along with ABI3, control nuclear size during seed maturation and germination in *Arabidopsis* (van Zanten et al. 2011). However, how plant CRWN proteins affect nuclear morphology, nuclear body formation and seed germination, remains to be elucidated. Here, we attempted to demonstrate the roles of *CRWN*s in seed germination. We revealed that *CRWN1* and *CRWN3*, which might genetically act upstream of *ABI5*, play pivotal roles in ABA-related seed germination by regulating the degradation of ABI5 protein in NBs, and the C-terminal of CRWN3 is necessary for its function.

RESULTS

Characterization of *crwn* mutants

In the CRWN family proteins, CRWN4 differs the most from the other CRWNs (Dittmer et al. 2007); therefore, we focused on CRWN1, CRWN2 and CRWN3 in this study. To investigate the functions of *CRWN* genes, we analyzed all the single and double mutant combinations of these genes during various developmental stages. The *crwn1*, *crwn2* and *crwn3* single mutants have no obvious growth defects throughout their lifetimes, consistent with previous reports (Dittmer et al. 2007; Wang et al. 2013). Among the three double mutants, *crwn1,3* plants had the most severely retarded growth phenotype, including short roots, small leaves, dwarf seedlings and short siliques (Figure 1A, B), whereas *crwn2,3* had a

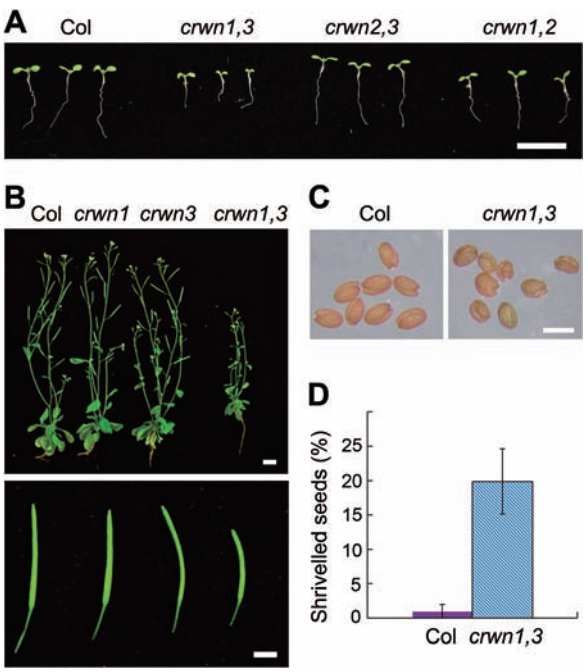

Figure 1. Characterization of *crwn* mutants

(**A**) Five-day-old seedlings of wild type (WT) and *crwn* double mutants. Bar, 1 cm. (**B**) Six-week-old seedlings (top) and siliques (bottom) of WT and *crwn* double mutants. Bar, 1 cm. (**C**) Seeds of WT and *crwn1,3* double mutants. Bar, 0.5 mm. (**D**) Rates of shriveled seeds of WT and *crwn1,3* double mutants. Data are mean values of three independent experiments ($n > 100$). Error bars represent standard deviation (*SD*).

wild type (WT)-like phenotype. The *crwn1,2* double mutant had a moderate phenotype, intermediate between *crwn1,3* and *crwn2,3* (Figure 1A). So we mainly focused on the *crwn1,3* double mutant hereafter. The *crwn1,3* mutants also produced about 10%–20% shriveled seeds, much higher than that of the WT (Figure 1C, D). The *crwn1,2,3* triple mutant combination is lethal and we did not obtain any triple mutants in the F2 progeny of the cross between the *crwn1,3* and *crwn2,3* plants, consistent with the results of Wang et al. (2013). These data suggested that *CRWN* genes play redundant and essential roles in plant growth and development.

crwn mutants are hypersensitive to ABA

To elucidate the functions of *CRWN1* and *CRWN3* in seed germination, we examined the germination rates of WT and *crwn* mutants in the absence and presence of exogenous ABA. In *Arabidopsis*, seed germination occurs in distinct stages, including testa rupture, endosperm rupture, radicle protrusion, cotyledon opening and cotyledon greening. Here, we used cotyledon greening as the marker of seed germination. On 1/2x Murashige and Skoog (MS) plates, the germination rates of *crwn1* and *crwn3* single mutants were comparable to that of WT (94.6%–97.0%), and the *crwn1,3* double mutant was slightly lower (61.3%, Figure 2). In the presence of 0.5 μmol/L ABA, the germination rate of WT decreased to 72.0%, whereas the germination of *crwn1* and *crwn3* seeds decreased to 55.7% and 57.7%, respectively. The germination rate of *crwn1,3* double mutant seed decreased dramatically, to 3.3% (Figure 2). These results indicated that the *crwn* mutants are hypersensitive to ABA and that *CRWN* genes play pivotal roles in ABA-dependent seed germination. We also examined other ABA-related phenotypes of the *crwn* mutants. In a root growth assay, the *crwn1,3* double mutant was hypersensitive to exogenous ABA, compared with WT (Figure S1). The expression of two ABI5 target genes, *ERD10* and *RD29A*, was slightly higher in the *crwn1,3* double mutant than that in WT in the absence of exogenous ABA. When treated with ABA, *ERD10* and *RD29A* were up-regulated more dramatically in *crwn1,3* than in WT, about 50-fold versus 10-fold and 200-fold versus 50-fold changes, respectively (Figure S2). This hypersensitivity of the *crwn1,3* double mutant to ABA suggested that *CRWN* genes have important roles in the ABA signaling pathway.

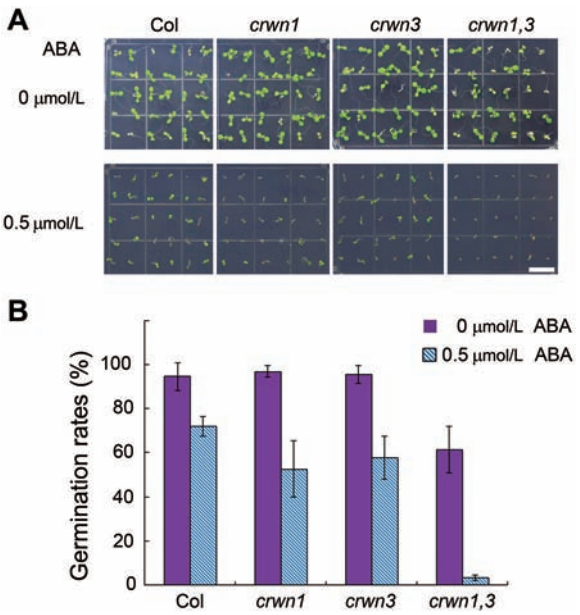

Figure 2. *crwn* mutants are hypersensitive to abiscisic acid in seed germination

(**A**) Phenotype of wild type (WT) and *crwn* mutants in seed germination. (**B**) Quantitative analysis of seed germination rates of WT and *crwn* mutants. The means of three independent experiments ($n > 100$) ± *SD* are shown.

CRWNs regulate ABI5 protein stability

Since ABI5 protein levels directly affect ABA-dependent seed germination and post-germination growth, we next explored the possible roles of *CRWNs* in regulation of ABI5 during seed germination. We first examined the ABI5 protein levels in WT and *crwn1,3* double mutants in the absence of exogenous ABA. In seeds germinated on 1/2x MS plates, at 1 d after germination (DAG) ABI5 protein levels in WT seeds decreased to about 13% of the protein levels at 0 DAG, and further decreased to undetectable levels at 2 DAG. By contrast, the *crwn1,3* seeds retained more than 50% of ABI5 protein at 1 DAG compared to the levels at 0 DAG, and also retained about 30% of ABI5 protein at 2 DAG (Figure 3A). We also checked the *ABI5* transcript levels in *crwn1,3* double mutants. In WT, the *ABI5* transcripts decreased to 45% at 1 DAG compared to the levels at 0 DAG. The *ABI5* transcripts in *crwn1,3* were about two-fold higher than that of WT at 0 DAG, and decreased by roughly half at 1 DAG, which were comparable to the WT 0 DAG level (Figure S3). These results suggested that CRWNs regulate ABI5 at both the messenger RNA (mRNA) and protein levels, but the protein-level regulation might play the major role.

ABA stabilizes ABI5 protein after seed germination (Lopez-Molina et al. 2001, 2003), so we examined ABI5 protein levels of WT and *crwn1,3* double mutant seed germinated on 1/2x MS plates containing 0.5 μmol/L ABA. We found that the ABI5 protein level in WT decreased obviously at 5 DAG, but the ABI5 protein in *crwn1,3* double mutants remained unaltered at 5 DAG (Figure 3B). This result suggested that CRWNs negatively regulate the ABA-dependent accumulation of ABI5 during seed germination, consistent with the ABA-hypersensitive phenotype of the *crwn1,3* double mutants (Figure 2). We also tested the ABI5 protein level after germination in the presence of cycloheximide (CHX), which blocks *de novo* protein synthesis. After CHX treatment, ABI5 protein decreased very rapidly in WT but remained stable in *crwn1,3* double mutants (Figure 3C). Previous work reported that ABI5 is degraded by the 26S proteasome pathway, so our results indicate that CRWN1 and CRWN3 might participate in the ubiquitin-mediated degradation of ABI5.

CRWNs function upstream of *ABI5* to regulate seed germination

The data presented above showed that *crwn* mutants are hypersensitive to ABA and have abnormal levels of ABI5 protein, indicating that *CRWNs* may genetically interact with key components in the ABA signaling pathway. To test this possibility, we constructed multiple mutants by crossing *abi5* and *crwn1,3* mutants. Under normal conditions, the seed

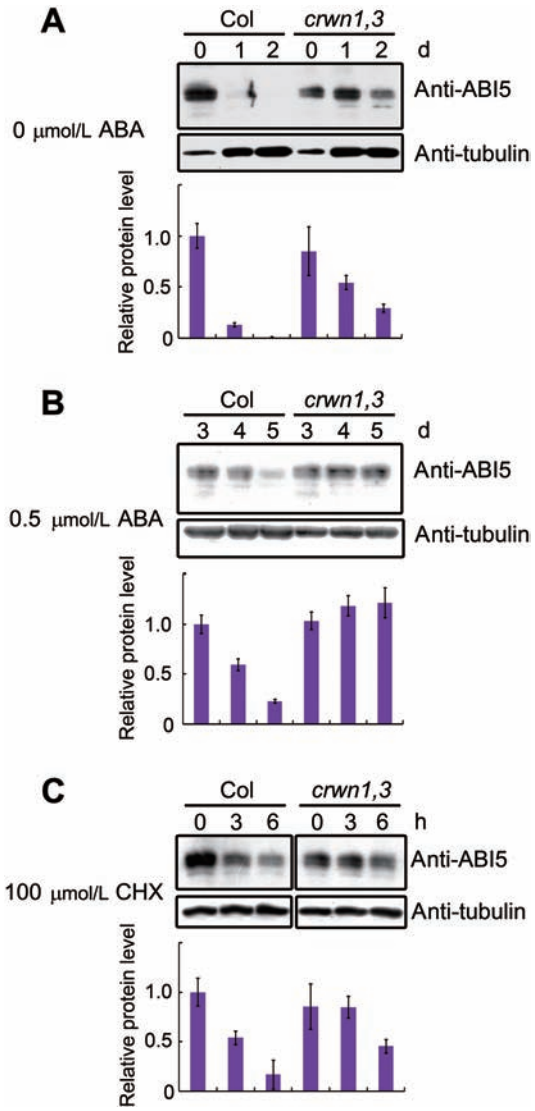

Figure 3. CRWN1 and CRWN3 regulate ABI5 protein stability
(**A**) ABI5 protein levels in wild type (WT) and *crwn1,3* double mutant seeds in the absence of exogenous abscisic acid (ABA). Seeds were stratified on 1/2x Murashige and Skoog (MS) plates at 4 °C in the dark for 2 d, and then transferred to 22 °C under continuous white light. Materials were collected at the indicated time for immunoblotting. (**B**) ABI5 protein levels in WT and *crwn1,3* double mutant seeds in the presence of exogenous ABA. Seeds were stratified on 1/2x MS plates containing 0.5 μmol/L ABA at 4 °C in the dark for 2 d, and then transferred to 22 °C under continuous white light. Materials were collected at the indicated time for immunoblotting. (**C**) The degradation of ABI5 protein in WT and *crwn1,3* double mutant. Col-0 and *crwn1,3* double mutant seeds were germinated on 1/2x MS with 0.5 μmol/L ABA for 3 d, and then treated with 100 μmol/L cycloheximide (CHX) in liquid 1/2x MS medium for the indicated times. Each lane contained about 20 μg total protein. An anti-tubulin immunoblot was used as a loading control. At the bottom of each panel, quantitative analysis of the relative level of the immunoblotting samples (the means of three independent experiments ± *SD*) is shown.

germination ratio of *abi5* mutants was similar to that of WT (98.8% vs. 96.0%), while the *crwn1,3* double mutant had a slightly lower ratio (61.3%). The germination ration of *crwn1,3 abi5* triple mutant (84.4%) resembled that of the *abi5* mutant (98.8%), rather than the *crwn1,3* double mutant (61.3%). In the presence of 0.5 μmol/L ABA, the germination rate of the *crwn1,3* double mutant seeds greatly increased in the *abi5* mutant background, from 3.0% to 75.0%, comparable to WT (Figure 4). This result suggests that *CRWN1* and *CRWN3* genetically act upstream of *ABI5*.

CRWN3 colocalized with ABI5 in nuclear bodies

ABI5 colocalizes with AFP and COP1 in NBs and is degraded there (Lopez-Molina et al. 2003). Since our results indicated that CRWN1 and CRWN3 were associated with ABI5 degradation, we speculated that ABI5 might colocalize with CRWN proteins in NBs. To test this hypothesis, we constructed *CRWN3-YFP* and *ABI5-mCherry* fusion genes under the control of a β-estradiol-inducible promoter and transformed them into WT plants and *crwn1,3* mutants. In WT roots treated with β-estradiol, 20% to 60% of CRWN-YFP protein displayed a punctate pattern in the nucleus and the remainder localized throughout the nucleoplasm (Figure 5A), consistent with a recent study (Sakamoto and Takagi 2013). In both WT and *crwn1,3* double mutants, ABI5-mCherry fusion protein localized exclusively in the nucleus but not in a punctate pattern, which is consistent with a previous report (Lopez-Molina et al. 2003). Interestingly, the ABI5-mCherry fluorescence signal showed a much higher density in the nuclei of *crwn1,3* double mutants than in WT plants (Figure 5B), in accordance with our observation that ABI5 protein is

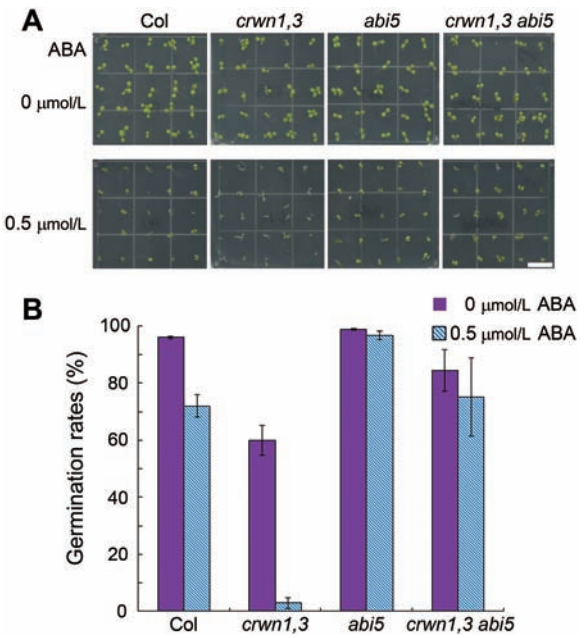

Figure 4. *CRWNs* function upstream of *ABI5* in seed germination
(**A**) Phenotype of wild type (WT), *abi5*, and *crwn* mutants in seed germination. (**B**) Quantitative analysis of seed germination rates of WT, *abi5* and *crwn* mutants. The means of three independent experiments ($n > 100$) ± *SD* are shown.

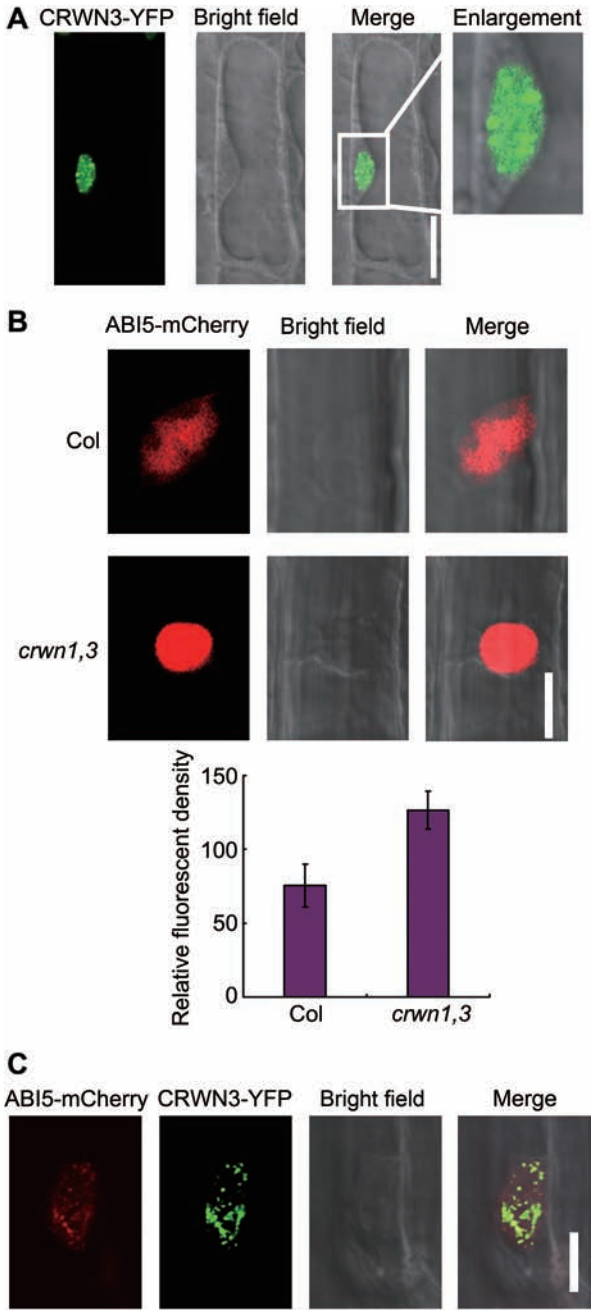

Figure 5. CRWN3 and ABI5 co-localize in nuclear bodies
(**A**) Subcellular localization of CRWN-YFP. Bar, 20 μm. (**B**) Subcellular localization of ABI5-mCherry in Col-0 and *crwn1,3* double mutant plants. Bar, 10 μm. The fluorescent signal was quantified using Image J ($n = 15$). (**C**) Colocalization of ABI5-mCherry and CRWN3-YFP in nuclear bodies. Bar, 10 μm.

more stable in *crwn1,3* double mutants than in WT (Figure 3A). The *crwn1,3* double mutants have noticeably smaller and more spherical nuclei than WT (Figure 5B), as also observed by Wang et al. (2013). This evidence confirmed the conclusion that CRWNs play important roles in control of nuclear size and shape.

Next, we transformed *CRWN3-YFP* and *ABI5-mCherry* fusion genes into *Arabidopsis* to examine whether these two proteins colocalized. We found that in more than 50% of the nuclei, YFP and mCherry fluorescence signals were overlapped and displayed a punctate distribution (Figure 5C), similar to the patterns of ABI5, AFP and COP1 (Lopez-Molina et al. 2003). It was worth noticing that when expressed alone, ABI5-mCherry protein localized throughout the nucleus (not a punctate distribution pattern, Figure 5B), whereas expressed alone with CRWN3-YFP, the two proteins colocalized in NBs (punctuate pattern, Figure 5C). This result indicates that CRWN3 might recruit ABI5 to NBs and regulate its degradation there.

CRWN3 function in the response to ABA requires the C-terminal domain

Arabidopsis CRWN proteins contain a large central coiled-coil domain and smaller N- and C-terminal domains. Although the N- and C-terminal domains have lower conservation on average, the last 12 amino acids are highly conserved in CRWN1, CRNW2 and CRWN3 (Dittmer et al. 2007; Meier et al. 2007), indicating that the extreme C terminus of CRWNs might play important roles. Indeed, the Val-1081 residue of CRWN3 is the only different site in the last 10 amino acids of the three CRWNs, as this site is Thr in CRWN1 and CRWN2 (refer to Dittmer et al. 2007, Figure 1C). To test the importance of the C-terminal region, we constructed a truncated version of *CRWN3* (*CRWN3*^{N1080}) that deletes the last five amino acids of the C terminal region, including Val-1081. We transformed *CRWN3*^{N1080}, as well as full-length *CRWN3*, into a *crwn1,3* double mutant. Either the truncated *CRWN3*^{N1080} or the WT *CRWN3* could complement the developmental defects and shriveled seeds of the *crwn1,3* double mutant under normal conditions (Figure 6A, B). Intriguingly, the ABI5 protein level in *CRWN3*^{N1080} plants was comparable to that in *crwn1,3* double mutants, which differed significantly from the ABI5 protein level in WT (Figures 3A, S4). In addition, the truncated version of *CRWN3* could not rescue the ABA response of *crwn1,3* double mutants during seed germination. In the absence of exogenous ABA, the germination rates of *CRWN3*^{N1080} and *CRWN3* transgenic plants (all in the *crwn1,3* double mutant background) are similar to those of the WT and *crwn1* mutant (≈97%). However, in the presence of 0.5 μmol/L ABA, the germination rate of *CRWN3* transgenic plants was comparable to that of *crwn1* single mutant (52.4% and 53.0%, respectively), whereas the germination rate of the *CRWN3*^{N1080} transgenic plants was comparable to the *crwn1,3* double mutant (15.0% and 10.0%, respectively), a germination rate much lower than that of *CRWN3* transgenic plants and *crwn1* mutants (Figure 6C, D). These results suggest that the extreme C-terminal of CRWN3 protein is necessary for its function in the response to ABA during seed germination, but not for normal post-germination growth.

DISCUSSION

In this study, we carried out a reverse genetic analysis of the function of *CRWN* genes in ABA-mediated seed germination. Our results showed that *crwn* mutants are hypersensitive to exogenous ABA, and the *abi5* mutation could suppress the

ABA sensitivity of *crwn* mutants in seed germination, which suggested that *CRWN*s act upstream of *ABI5*. Moreover, the ABI5 protein, which is directly correlated with ABA-dependent seed germination, is more stable in *crwn* mutants than in WT. This evidence indicated that *CRWN* genes may regulate seed germination by modulating the ABA signaling pathway. Supporting this conclusion, van Zanten et al. (2011) recently reported that *CRWN1*, *CRWN2* and *ABI3*, another key component of the ABA signaling pathway, affect nuclear size during seed maturation and germination, with *ABI3* mediating a reduction in nuclear size and *CRWN1* and *CRWN2* mediating an increase in nuclear size. *ABI3* encodes a B3 transcription factor and also acts upstream of *ABI5* by regulating its mRNA level (Lopez-Molina et al. 2002); the relationship between *CRWN*s and *ABI3* will be an interesting question for future study. They may regulate ABI5 in the same or independent pathways. Our analyses also confirmed the functional redundancy and necessity of *CRWN* genes with regard to their effects on whole-plant morphology, nuclear size and viability, as reported in previous studies (Dittmer et al. 2007; Sakamoto and Takagi 2013; Wang et al. 2013).

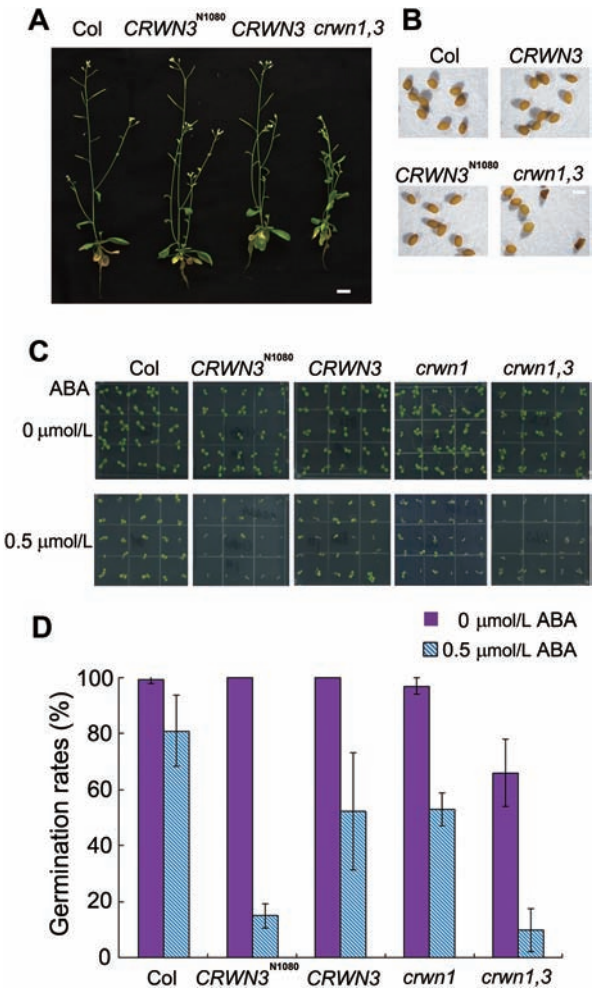

Figure 6. The C-terminal of CRWN3 is essential for its function in the response to ABA

(**A**) Phenotype of 6-week-old seedlings. Bar, 1 cm. (**B**) Seeds of wild type (WT), *CRWN* complemented plants and *crwn1,3* double mutants. Bar, 0.5 mm. (**C**) Phenotype of WT, *CRWN* complemented plants and *crwn* mutants in seed germination. (**D**) Quantitative analysis of seed germination rates of WT, *CRWN* complemented plants and *crwn* mutants. The means of three independent experiments ($n > 100$) $\pm$ *SD* are shown. The transgenic plants *CRWN3*^{N1080} and *CRWN3* represent *crwn1,3* double mutants carrying a truncated and a full-length version of the *CRWN3* transgene, respectively. For each gene, more than 20 transgenic lines were generated and 10 of them were carefully analyzed for their phenotype and response to abscisic acid (ABA), and similar results were obtained.

ABI5 protein was reported to be degraded through the 26S proteasome pathway (Lopez-Molina et al. 2001; Stone et al. 2006; Lee et al. 2010; Liu and Stone 2010). The colocalization of ABI5, AFP and COP1 in NBs indicated that ABI5 degradation occurs there (Lopez-Molina et al. 2003). We observed that CRWN3 colocalized with ABI5 in NBs, which raised the possibility of the involvement of CRWN3 in ABI5 protein degradation. Three lines of evidence support this idea. First, the ABI5 protein was more stable in *crwn1,3* mutants than in WT plants. Second, the density of ABI5-mCherry fluorescent signal was much higher in *crwn1,3* mutants than in WT, and the distribution of ABI5 also seemed to be altered in *crwn1,3*. Third, ABI5-mCherry protein localized throughout the nucleus when expressed alone, and could colocalize with CRWN3-YFP in a punctate distribution pattern when expressed alone with it (Figure 5). So, we propose that CRWNs might recruit ABI5 to NBs and regulate its degradation there. We attempted to examine whether ABI5 protein directly binds to CRWN3 by using yeast-two hybrid and co-immunoprecipitation assays, but could not detect an interaction (data not shown). We therefore suggest that a linker protein may mediate the interaction between CRWN3 and ABI5. Isolating and identifying the proteins that interact with CRWN3 and ABI5 will be key to improving our understanding of the molecular mechanism of ABA-regulated seed germination. In *Arabidopsis*, there are distinct E3 ligase (complex)-related ABI5 degradation mechanisms, which may function in different developmental stages or growth conditions. For example, ABI5 protein accumulates to elevated levels in *dwa* mutants compared to WT after ABA and NaCl treatments, but shows no difference between *dwa* mutants and WT under normal growth conditions (Lee et al. 2010). However, *keg* mutants have much higher ABI5 protein levels than WT under standard conditions (Stone et al. 2006; Liu and Stone 2010). The situation with the *crwn* mutants is similar to that of *keg*, so we speculate that CRWNs might be involved in KEG-mediated ABI5 degradation. The relationship between CRWNs and KEG should be studied further to dissect the mechanism by which CRWNs regulate ABI5 stability.

Despite many observations of phytochrome receptor NBs and phytohormone-related NBs, the mechanism of NB organization in plants remains unknown. In mammalian cells, the initiation of NBs requires a coiled-coil domain-containing protein, such as PML, conjugating with nuclear matrix proteins, such as lamin A, which also have coiled-coil domains, in the nucleus (Müller et al. 1998; Lallemand-Breitenbach et al. 2001). Coiled-coil domains generally act as protein-protein interaction motifs and coiled-coil proteins function in diverse biological processes in eukaryotic organisms, such as

cyto- and nucleoskeleton organization, spindle-pole and centrosome formation and membrane structure (Burkhard et al. 2001; Rose and Meier 2004). In a plants, few coiled-coil proteins, including kinesin-like proteins, SMC-like proteins and NMCP, are reported to be involved in cytokinesis, DNA repair, seed development and spindle organization, respectively (Masuda et al. 1997; Mengiste et al. 1999; Hanin et al. 2000; Strompen et al. 2002; Nishihama et al. 2002; Marcus et al. 2003).

In this study, we examined the roles of *Arabidopsis* CRWN proteins, which typically contain a long coiled-coil motif, in ABI5 NB formation during seed germination. We found that CRWN3 colocalized with ABI5 in NBs where ABI5 protein may be degraded. Another group also observed the punctate distribution of CRWN3 (Sakamoto and Takagi 2013). This result indicates that the predicted nuclear matrix protein CRWN3 is important for the formation of ABI5 NBs. We also cannot exclude possible roles of other CRWNs in NB organization. One interesting finding in our study was that the truncated $CRWN3^{N1080}$ could rescue the developmental defects of *crwn1,3* double mutants, but not the ABA response during seed germination. The large coiled-coil domains in the central region of all four CRWNs are highly conserved, whereas the small N- and C-terminal regions of these CRWNs have less similarity (Dittmer et al. 2007; Meier et al. 2007). So, we suppose that the conserved coiled-coil domains of CRWNs serve general roles in protein-protein interaction and basic structure in plant cells, and the C-terminal region, possibly just several amino acids, plays specific roles in signal integration, such as during ABA-controlled seed germination.

We note that the process of PML NB formation in mammalian cells has strong similarities to the process of ABI5 NB formation in plant cells. For PML NB organization, dephosphorylation of PML targets it to the nuclear matrix to form the primary PML NB. The subsequent sumolation of PML induces the maturation to secondary PML NBs, where PML will be degraded (Lallemand-Breitenbach et al. 2001). Analogously, ABI5 protein can also be phosphorylated, dephosphorylated, sumolated and ubiquitinated. One attractive model is that ABI5 could be anchored to nuclear matrix proteins, such as CRWNs, upon its dephosphorylation, then ubiquitinated and finally degraded in NBs. This hypothesis is supported by the fact that phosphorylation makes ABI5 protein more stable, enabling it to regulate the ABA signaling pathway (Finkelstein and Lynch 2000; Lopez-Molina et al. 2001). Nevertheless, this working model requires more evidence.

In mammalian cells, PML NBs recruit an astonishing variety of seemingly unrelated proteins (Lallemand-Breitenbach and de The 2010). We also noticed that many plant proteins colocalize in NBs. For example, phyB NBs contain CRY2 in a tobacco transient transformation system (Más et al. 2000). Some positive regulators of the light signaling pathway, including HY5, LAF1, HFR1, PIF1 and PIF3, colocalized with COP1 in NBs (Al-Sady et al. 2006; Chen et al. 2010; Van Buskirk et al. 2012). COP1, an important component of the E3 ligase complex and light signal transduction pathway, colocalizes with ABI5, AFP and SIZ1 in NBs (Lopez-Molina et al. 2003; Miura et al. 2005, 2009). Our results showed that CRWN3 localized with ABI5, and hence likely localized with AFP, COP1 and possibly with some components of phytochrome NBs. The large numbers and types of proteins aggregating in NBs may indicate that the NB-related regulation functions as a general mechanism that controls biological processes in eukaryotes. As the functionally conserved nuclear matrix proteins, CRWNs, similar to their counterparts in animals, are necessary for formation of NBs. The colocalization of light and ABA signaling pathway components in NBs highlights the possibility that light and phytohormone signals might converge in NBs. Identification and characterization of more components of ABI5 NBs will help to elucidate the mechanisms through which these two signals crosstalk.

MATERIALS AND METHODS

Plant materials and growth conditions

The wild-type *Arabidopsis thaliana* L. Col-0 accession was used in this study. All mutants used in this study, including *crwn1-1* (SALK_025347), *crwn2-1* (SALK_076653), *crwn3-2* (SALK_094835) and *abi5-8* (SALK_013163), are in the Col-0 background and were obtained from ABRC. For the multiple mutant analysis, the segregated wild-type and single mutants progenies in F2 or F3 populations were used as controls. Transgenic *Arabidopsis* lines were generated by the *Agrobacterium tumefaciens*-mediated floral dip method (Clough and Bent 1998), using strain GV3101.

Genotyping of the T-DNA insertion lines was performed using the following pairs of gene-specific primers: SALK_025347 (*crwn1-1*) RP 5′-AGT TTC CAA TGC CTT CTC CTC-3′ and LP1 5′-AGT TCT CTT CAA AGG GAG CG-3′; SALK_076653 (*crwn2-1*) LP 5′-CTC GAA CTG AGC CAT TCT GTC-3′ and RP 5′-AGC TCA TTG CTA GAG AAG GGG-3′; SALK_094835 (*crwn3-2*) LP 5′-AGA AAG AGT GGG AAG CTT TGG-3′ and RP 5′-TTT TCC TAC GTC CAC CAG TTG-3′; SALK_013163 (*abi5-8*) LP 5′-CAA TGG AAG TTC GGA ATC ATG-3′ and RP 5′-CAC TCG TTT TCT TCT TAA AGC G-3′.

Seeds surface-sterilized with 70% (V/V) ethanol were stratified in the dark at 4 °C for 2 d, and germinated in soil or on 1/2x MS medium containing 1% sucrose and 0.05% MES-KOH, and grown at 22 °C under continuous white light (120 mmol/m^2/s) for the appropriate time.

Seed germination assay

The same batches of seeds of various genotypes grown under the same conditions were used for the seed germination assay. After 2 d of stratification in the dark at 4 °C, seeds were transferred to a tissue culture room at 22 °C under continuous white light. Seed germination was scored at 4 d after transferring from darkness to light. The mean values obtained from three independent batches of seeds (biological repeats) are shown.

Molecular manipulations

All molecular manipulations were done by standard methods (Sambrook and Russell 2001). For observing the localization of CRWN3 and ABI5, full-length coding sequences (CDS) of *CRWN3* and *ABI5* without stop codons were amplified by reverse transcription polymerase chain reaction (RT-PCR) with primers *CRWN3*-F12 (5′-CCT GTC GAC ATG TTC ACT CCG CAA AGG AAT C-3′)/*CRWN3*-B11 (5′-AGT CCC GGG TGT TGT GAA AAA GAC CCA AAT CT-3′), and ABI5-F13 (5′-TCA CTC GAG ATG GTA

ACT AGA GAA ACG AAG-3′)/ABI5-B11 (5′-TCA GAT ATC GAG TGG ACA ACT CGG GTT CC-3′), respectively. Both of these genes were controlled by a β-estradiol-inducible promoter. *CRWN3* fragments were fused in-frame to the YFP coding sequence and then inserted into the *Apa*I and *Spe*I sites of the binary vector pER8. *ABI5* fragments were fused to the mCherry CDS and cloned between the *Xho*I and *Spe*I sites of the pER10 vector (Zuo et al. 2000). For complementation assays, *CRWN3* and *CRWN3*^{N1080} CDS were amplified with primers *CRWN3*-F (5′-AGT GTC GAC ATG GAG ATT GGG AAG CTT CTT G-3′)/*CRWN3*-B (5′-TCA TGT TGT GAA AAA GAC C-3′), and *CRWN3*-F/*CRWN3*-B18 (5′-AGT CCC GGG TCA CCA AAT CTT TTT TCC TAT-3′), respectively. The CDS were driven by the *CRWN3* promoter, which was amplified with primers *CRWN3*-PF (5′-ACC GGG CCC GTA GGA GTA TTA GTT TAA TA-3′)/*CRWN3*-PB (5′-AGT GTC GAC TTC CGT TCG AAC CCT AGA-3′). The promoter and CDS were cloned into a modified pER8 vector between the *Apa*I and *Sma*I sites.

Protein isolation and immunoblot analysis

Plant proteins were isolated with RIPA buffer containing 50 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 0.1% Nonidet P-40, 0.5% sodium deoxycholate, 1% sodium dodecyl sulfate and 1X protein inhibitor cocktail (Sigma–Aldrich, Cat# P9599). Protein immunoblotting was performed as previously described (Ren et al. 2009), by using anti-ABI5 (Guan et al. 2014) and anti-tubulin antibodies (Sigma–Aldrich, Cat# T8203).

Real-time RT-qPCR

Total RNA was prepared using the RNeasy plant mini kit (Qiagen) and then treated with RNase-free DNase (Qiagen) for 15 min at room temperature. The first strand of cDNA was synthesized using TransScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech), and then used as the template for RT quantitative PCR (RT-qPCR). The relative expression level of each target gene was normalized with that of *ACTIN7*. Real-time RT-qPCR was performed with primers ABI5 RT-F (5′-GCG GGT GGA CAG CAA ATG GGA ATG-3′)/ABI5 RT-R (5′-ATG CTT GTT TTC TTG CTC TAG ATC-3′), ERD10 RT-F (5′-CAA GCC AAG TCT CCT CGA CAA-3′)/ERD10 RT-R (5′-ATC CTG TCC ATT ACT CCT TGA-3′), RD29A RT-F (5′-CAC ACA CCA GCA GCA CCC AG-3′)/RD29A RT-R (5′-CTG GTG CAT CGA TCA CTT CAG-3′) and Actin7 RT-F (5′-GGA ACT GGA ATG GTG AAG GCT G-3′)/Actin7 RT-R (5′-CGA TTG GAT ACT TCA GAG TGA GGA-3′). Real-time PCR was performed using the UltraSYBR mixture (CWBIO) according to the manufacturer's instructions, and the reactions were run in a CFX96TM real-time PCR detection system (Bio-Rad).

Confocal microscopy

Transgenic plants were grown on 1/2x MS medium for 8 d and transferred to 1/2x MS plates containing 10 μmol/L β-estradiol for 12 h, then YFP and mCherry fluorescence in roots were observed with an Olympus Fluoview1000 confocal laser scanning microscope.

Accession numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *CRWN1* (At1G67230), *CRWN2* (At1G13220), *CRWN3* (At1G68790) and *ABI5* (At2G36270).

ACKNOWLEDGEMENTS

We thank *Arabidopsis* Biological Resource Center and Dr. Yan Guo for providing mutant seeds. This work was supported by grants from the National Natural Science Foundation (31100211) and the Ministry of Science and Technology of China (2014CB943402).

AUTHOR CONTRIBUTIONS

W.Z., B.R., and J.Z. designed the research and analyzed the results. W.Z. and B.R. wrote the article. W.Z. performed the majority of the experiments, assisted by B.R., C.G., J.F., Y.L. and N.Z.

REFERENCES

Al-Sady B, Ni WM, Kircher S, Schafer E, Quail PH (2006) Photoactivated phytochrome induces rapid PIF3 phosphorylation prior to proteasorne-mediated degradation. **Mol Cell** 23: 439–446

Bewley JD (1997) Seed germination and dormancy. **Plant Cell** 9: 1055–1066

Brocard IM, Lynch TJ, Finkelstein RR (2002) Regulation and role of the *Arabidopsis* abscisic acid-insensitive 5 gene in abscisic acid, sugar, and stress response. **Plant Physiol** 129: 1533–1543

Burkhard P, Stetefeld J, Strelkov SV (2001) Coiled coils: A highly versatile protein folding motif. **Trends Cell Biol** 11: 82–88

Catala R, Ouyang J, Abreu IA, Hu Y, Seo H, Zhang X, Chua NH (2007) The *Arabidopsis* E3 SUMO ligase SIZ1 regulates plant growth and drought responses. **Plant Cell** 19: 2952–2966

Chen M, Galvao RM, Li M, Burger B, Bugea J, Bolado J, Chory J (2010) *Arabidopsis* HEMERA/pTAC12 initiates photomorphogenesis by phytochromes. **Cell** 141: 1230–1237

Clough SJ, Bent AF (1998) Floral dip: A simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. **Plant J** 16: 735–743

Daniel MT, Koken M, Romagne O, Barbey S, Bazarbachi A, Stadler M, Guillemin MC, Degos L, Chomienne C, de The H (1993) PML protein expression in hematopoietic and acute promyelocytic leukemia cells. **Blood** 82: 1858–1867

Dekkers BJ, Schuurmans JA, Smeekens SC (2008) Interaction between sugar and abscisic acid signalling during early seedling development in *Arabidopsis*. **Plant Mol Biol** 67: 151–167

Dittmer TA, Stacey NJ, Sugimoto-Shirasu K, Richards EJ (2007) *LITTLE NUCLEI* genes affecting nuclear morphology in *Arabidopsis thaliana*. **Plant Cell** 19: 2793–2803

Dyck JA, Maul GG, Miller WH, Chen JD, Kakizuka A, Evans RM (1994) A novel macromolecular structure is a target of the promyelocyte-retinoic acid receptor oncoprotein. **Cell** 76: 333–343

Favory JJ, Stec A, Gruber H, Rizzini L, Oravecz A, Funk M, Albert A, Cloix C, Jenkins GI, Oakeley EJ (2009) Interaction of COP1 and UVR8 regulates UV-B-induced photomorphogenesis and stress acclimation in *Arabidopsis*. **EMBO J** 28: 591–601

Finkelstein RR (1994) Mutations at two new *Arabidopsis* ABA response loci are similar to the *abi3* mutations. **Plant J** 5: 765–771

Finkelstein RR, Lynch TJ (2000) The *Arabidopsis* abscisic acid response gene *ABI5* encodes a basic leucine zipper transcription factor. **Plant Cell** 12: 599–609

Finkelstein RR, Wang ML, Lynch TJ, Rao S, Goodman HM (1998) The *Arabidopsis* abscisic acid response locus *ABI4* encodes an APETALA2 domain protein. **Plant Cell** 10: 1043–1054

Gu NN, Zhang YC, Yang HQ (2012) Substitution of a conserved glycine in the PHR domain of *Arabidopsis* CRYPTOCHROME 1 confers a constitutive light response. **Mol Plant** 5: 85–97

Guan C, Wang X, Feng J, Hong S, Liang Y, Ren B, Zuo J (2014) Cytokinin antagonizes abscisic acid-mediated inhibition of cotyledon greening by promoting the degradation of abscisic acid insensitive5 protein in *Arabidopsis*. **Plant Physiol** 164: 1515–1526

Hanin M, Mengiste T, Bogucki A, Paszkowski J (2000) Elevated levels of intrachromosomal homologous recombination in *Arabidopsis* overexpressing the *MIM* gene. **Plant J** 24: 183–189

Karssen C, Zagorski S, Kepczynski J, Groot S (1989) Key role for endogenous gibberellins in the control of seed germination. **Ann Bot** 63: 71–80

Karssen CM, Brinkhorst-van der Swan DL, Breekland AE, Koornneef M (1983) Induction of dormancy during seed development by endogenous abscisic acid: Studies on abscisic acid deficient genotypes of *Arabidopsis thaliana* (L.) Heynh. **Planta** 157: 158–165

Kircher S, Gil P, Kozma-Bognár L, Fejes E, Speth V, Husselstein-Muller T, Bauer D, Ádám É, Schäfer E, Nagy F (2002) Nucleocytoplasmic partitioning of the plant photoreceptors phytochrome A, B, C, D, and E is regulated differentially by light and exhibits a diurnal rhythm. **Plant Cell** 14: 1541–1555

Kleiner O, Kircher S, Harter K, Batschauer A (1999) Nuclear localization of the *Arabidopsis* blue light receptor cryptochrome 2. **Plant J** 19: 289–296

Kobayashi Y, Murata M, Minami H, Yamamoto S, Kagaya Y, Hobo T, Yamamoto A, Hattori T (2005) Abscisic acid-activated SNRK2 protein kinases function in the gene-regulation pathway of ABA signal transduction by phosphorylating ABA response element-binding factors. **Plant J** 44: 939–949

Koken MH, Puvion-Dutilleul F, Guillemin MC, Viron A, Linares-Cruz G, Stuurman N, de Jong L, Szostecki C, Calvo F, Chomienne C, Degos L, Puvion E, de The H (1994) The t(15;17) translocation alters a nuclear body in a retinoic acid-reversible fashion. **EMBO J** 13: 1073–1083

Koornneef M, Reuling G, Karssen C (1984) The isolation and characterization of abscisic acid-insensitive mutants of *Arabidopsis thaliana*. **Physiol Plant** 61: 377–383

Kucera B, Cohn MA, Leubner-Metzger G (2005) Plant hormone interactions during seed dormancy release and germination. **Seed Sci Res** 15: 281–307

Lallemand-Breitenbach V, de The H (2010) PML nuclear bodies. **Cold Spring Harb Perspect Biol** 2: a000661

Lallemand-Breitenbach V, Zhu J, Puvion F, Koken M, Honore N, Doubeikovsky A, Duprez E, Pandolfi PP, Puvion E, Freemont P, de The H (2001) Role of promyelocytic leukemia (PML) sumolation in nuclear body formation, 11S proteasome recruitment, and As_2O_3-induced PML or PML/retinoic acid receptor alpha degradation. **J Exp Med** 193: 1361–1371

Le, JH, Yoon HJ, Terzaghi W, Martinez C, Dai M, Li J, Byun MO, Deng XW (2010) DWA1 and DWA2, two *Arabidopsis* DWD protein components of CUL4-based E3 ligases, act together as negative regulators in ABA signal transduction. **Plant Cell** 22: 1716–1732

Lian H, He S, Zhang Y, Zhu D, Zhang J, Jia K, Sun S, Li L, Yang HQ (2011) Blue-light-dependent interaction of cryptochrome 1 with SPA1 defines a dynamic signaling mechanism. **Genes Dev** 25: 1023–1028

Liu B, Zuo Z, Liu H, Liu X, Lin C (2011) *Arabidopsis* cryptochrome 1 interacts with SPA1 to suppress COP1 activity in response to blue light. **Genes Dev** 25: 1029–1034

Liu H, Stone SL (2010) Abscisic acid increases *Arabidopsis* ABI5 transcription factor levels by promoting KEG E3 ligase self-ubiquitination and proteasomal degradation. **Plant Cell** 22: 2630–2641

Lopez-Molina L, Chua NH (2000) A null mutation in a bZIP factor confers ABA-insensitivity in *Arabidopsis thaliana*. **Plant Cell Physiol** 41: 541–547

Lopez-Molina L, Mongrand S, Chua NH (2001) A postgermination developmental arrest checkpoint is mediated by abscisic acid and requires the ABI5 transcription factor in *Arabidopsis*. **Proc Natl Acad Sci USA** 98: 4782–4787

Lopez-Molina L, Mongrand S, Kinoshita N, Chua NH (2003) AFP is a novel negative regulator of ABA signaling that promotes ABI5 protein degradation. **Genes Dev** 17: 410–418

Lopez-Molina L, Mongrand S, McLachlin DT, Chait BT, Chua NH (2002) ABI5 acts downstream of ABI3 to execute an ABA-dependent growth arrest during germination. **Plant J** 32: 317–328

Müller S, Matunis MJ, Dejean A (1998) Conjugation with the ubiquitin-related modifier SUMO-1 regulates the partitioning of PML within the nucleus. **EMBO J** 17: 61–70

Más P, Devlin PF, Panda S, Kay SA (2000) Functional interaction of phytochrome B and cryptochrome 2. **Nature** 408: 207–211

Marcus AI, Li W, Ma H, Cyr RJ (2003) A kinesin mutant with an atypical bipolar spindle undergoes normal mitosis. **Mol Biol Cell** 14: 1717–1726

Masuda K, Xu ZJ, Takahashi S, Ito A, Ono M, Nomura K, Inoue M (1997) Peripheral framework of carrot cell nucleus contains a novel protein predicted to exhibit a long α-helical domain. **Exp Cell Res** 232: 173–181

Meier I (2007) Composition of the plant nuclear envelope: Theme and variations. **J Exp Bot** 58: 27–34

Mengiste T, Revenkova E, Bechtold N, Paszkowski J (1999) An SMC-like protein is required for efficient homologous recombination in *Arabidopsis*. **EMBO J** 18: 4505–4512

Miura K, Lee J, Jin JB, Yoo CY, Miura T, Hasegawa PM (2009) Sumoylation of ABI5 by the *Arabidopsis* SUMO E3 ligase SIZ1 negatively regulates abscisic acid signaling. **Proc Natl Acad Sci USA** 106: 5418–5423

Miura K, Rus A, Sharkhuu A, Yokoi S, Karthikeyan AS, Raghothama KG, Baek D, Koo YD, Jin JB, Bressan RA (2005) The *Arabidopsis* SUMO E3 ligase SIZ1 controls phosphate deficiency responses. **Proc Natl Acad Sci USA** 102: 7760–7765

Nambara E, Marion-Poll A (2005) Abscisic acid biosynthesis and catabolism. **Annu Rev Plant Biol** 56: 165–185

Nishihama R, Soyano T, Ishikawa M, Araki S, Tanaka H, Asada T, Irie K, Ito M, Terada M, Banno H, Yamazaki Y, Machida Y (2002) Expansion of the cell plate in plant cytokinesis requires a kinesin-like protein/MAPKKK complex. **Cell** 109: 87–99

Parcy F, Valon C, Raynal M, Gaubier-Comella P, Delseny M, Giraudat J (1994) Regulation of gene expression programs during *Arabidopsis* seed development: Roles of the *ABI3* locus and of endogenous abscisic acid. **Plant Cell** 6: 1567–1582

Penfield S, Josse EM, Kannangara R, Gilday AD, Halliday KJ, Graham IA (2005) Cold and light control seed germination through the bHLH transcription factor SPATULA. **Curr Biol** 15: 1998–2006

Ren B, Liang Y, Deng Y, Chen Q, Zhang J, Yang X, Zuo J (2009) Genome-wide comparative analysis of type-A *Arabidopsis* response regulator genes by overexpression studies reveals their diverse roles and regulatory mechanisms in cytokinin signaling. **Cell Res** 19: 1178–1190

Rose A, Meier I (2004) Scaffolds, levers, rods and springs: Diverse cellular functions of long coiled-coil proteins. **Cell Mol Life Sci** 61: 1996–2009

Sakamoto Y, Takagi S (2013) LITTLE NUCLEI 1 and 4 regulate nuclear morphology in *Arabidopsis thaliana*. **Plant Cell Physiol** 54: 622–633

Sambrook J, Russell DW (2001) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Shaw PJ, Brown JW (2004) Plant nuclear bodies. **Curr Opin Plant Biol** 7: 614–620

Spector DL (2006) SnapShot: Cellular bodies. **Cell** 127: 1071

Stone SL, Williams LA, Farmer LM, Vierstra RD, Callis J (2006) KEEP ON GOING, a RING E3 ligase essential for *Arabidopsis* growth and development, is involved in abscisic acid signaling. **Plant Cell** 18: 3415–3428

Strompen G, El Kasmi F, Richter S, Lukowitz W, Assaad FF, Jurgens G, Mayer U(2002) The *Arabidopsis HINKEL* gene encodes a kinesin-related protein involved in cytokinesis and is expressed in a cell cycle-dependent manner. **Curr Biol** 12: 153–158

Tao L, Cheung AY, Nibau C, Wu H (2005) RAC GTPases in tobacco and *Arabidopsis* mediate auxin-induced formation of proteolytically active nuclear protein bodies that contain AUX/IAA proteins. **Science's STKE** 17: 2369–2384

Umezawa T, Sugiyama N, Mizoguchi M, Hayashi S, Myouga F, Yamaguchi-Shinozaki K, Ishihama Y, Hirayama T, Shinozaki K (2009) Type 2C protein phosphatases directly regulate abscisic acid-activated protein kinases in *Arabidopsis*. **Proc Natl Acad Sci USA** 106: 17588–17593

van Buskirk EK, Decker PV, Chen M (2012) Photobodies in light signaling. **Plant Physiol** 158: 52–60

van Zanten M, Koini MA, Geyer R, Liu Y, Brambilla V, Bartels D, Koornneef M, Fransz P, Soppe WJJ (2011) Seed maturation in *Arabidopsis thaliana* is characterized by nuclear size reduction and increased chromatin condensation. **Proc Natl Acad Sci USA** 108: 20219–20224

Wang H, Dittmer TA, Richards EJ (2013) *Arabidopsis* CROWDED NUCLEI (CRWN) proteins are required for nuclear size control and heterochromatin organization. **BMC Plant Biol** 13: 200

Wang H, Ma LG, Li JM, Zhao HY, Deng XW (2001) Direct interaction of *Arabidopsis* cryptochromes with COP1 in light control development. **Sci Signal** 294: 154–158

Weis K, Rambaud S, Lavau C, Jansen J, Carvalho T, Carmo-Fonseca M, Lamond A, Dejean A (1994) Retinoic acid regulates aberrant nuclear localization of PML-RAR alpha in acute promyelocytic leukemia cells. **Cell** 76: 345–356

Yamaguchi R, Nakamura M, Mochizuki N, Kay SA, Nagatani A (1999) Light-dependent translocation of a phytochrome B-GFP fusion protein to the nucleus in transgenic *Arabidopsis*. **J Cell Biol** 145: 437–445

Yu X, Sayegh R, Maymon M, Warpeha K, Klejnot J, Yang H, Huang J, Lee J, Kaufman L, Lin C (2009) Formation of nuclear bodies of *Arabidopsis* CRY2 in response to blue light is associated with its blue light–dependent degradation. **Plant Cell** 21: 118–130

Zuo Z, Liu H, Liu B, Liu X, Lin C (2011) Blue light-dependent interaction of CRY2 with SPA1 regulates COP1 activity and floral initiation in *Arabidopsis*. **Curr Biol** 21: 841–847

Zuo J, Niu Q, Chua N (2000) Technical advance: An estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. **Plant J** 24: 265–273

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Figure S1. Root growth phenotype of WT and *crwn1,3* double mutant plants

WT (Col) and *crwn1,3* double mutant seeds were germinated on 1/2x MS plates vertically for 7 d, then transferred to 1/2x MS plates with or without 20 μmol/L ABA and invertible grown for another 5 d. The experiment was repeated three times and a representative result is shown.

Figure S2. Analysis of the expression level of *ERD10* and *RD29A* in WT and *crwn1,3* mutant plants

One DAG seeds of Col and *crwn1,3* plants were treated with or without 20 mmol/L ABA for 2 h. Data presented are mean values of three independent experiments. Error bars denote *SD*.

Figure S3. Analysis of the expression level of *ABI5* in WT and crwn1,3 mutant plants

Col and *crwn1,3* seeds were germinated on 1/2x MS plates and incubated for the indicated times. Data presented are mean values of three independent experiments. Error bars denote *SD*.

Figure S4. ABI5 protein levels in *crwn1,3* and $CRWN3^{N1080}$ plants

The *crwn1,3* mutants and $CRWN^{1080N}$ transgenic seeds were germinated on 1/2x MS plates and incubated for the indicated times. The experiment was repeated three times and a representative result is shown.